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Genetic differentiation in the endangered myrmecophilous butterfly *Niphanda fusca*: a comparison of natural and secondary habitats

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Abstract

Niphanda fusca is an endangered myrmecophilous butterfly inhabiting environments at early stages of succession. Most of its habitats are places where succession is prevented by human activity. In some places, however, *N. fusca* lives in natural semi-bare areas, such as cliffs in mountains or grasslands around volcanos. We investigate the genetic structure of *N. fusca* in Japan and South Korea to address two questions. 1) Are populations in natural environments genetically different from those in secondary environments? and 2) Do populations in natural environments possess greater genetic diversity than those in secondary environments? The AMOVA results indicated that the populations in natural environments and those in secondary environments were differentiated to some extent; however, more than 80% of genetic variation was found to occur within the same habitat type and within each population. We found no differences in genetic diversity between populations in the two environments. At present, we have not found a strong reason to consider populations in the two environments as different evolutionarily significant units. We think it is practical to conserve populations in natural environments at first, because in this case we need not manage habitats to protect *N. fusca*. We have only to inhibit habitat destruction. In contrast, in order to conserve populations in secondary environments, we would have to continue managing the habitats. This is far more difficult than inhibiting habitat destruction.

Keywords: ant, genetic structure, mitochondrial DNA, nuclear DNA, parasite

Introduction

Population fragmentation enhances the risk of extinction through loss of genetic diversity via drift, inbreeding, and local adaptation (Frankham et al. 2002). Human activities are one of the greatest threats to population fragmentation of natural organisms (e.g., Laurance et al. 2000; Pimm SL et al. 2014). However, some life history traits of natural organisms would also lead to population fragmentation. To what extent populations experience fragmentation varies depending on the species.

The majority of lycaenid butterflies have associations with ants that can be facultative or obligate, and range from mutualism to parasitism (Pierce et al. 2002). Some species are obligate parasites: they live in ant nests where they are fed mouth-to-mouth by the adult ants or eat the ant brood (Pierce et al. 2002). Associations between butterflies and ants have attracted the attention of many biologists because they provide an ideal opportunity to study symbiotic relationships (e.g., Als et al. 2004; Eastwood et al. 2006; Nash et al. 2008). Myrmecophilous butterflies have other notable characteristics. In butterflies that have obligate association with ants, overlapping

requirements of suitable host plants and attendant ants can lead to population fragmentation, thus promoting genetic divergence among populations. Such population fragmentation must also increase the risk of local extinction (Frankham et al. 2002). In fact, obligate myrmecophilous butterflies have seriously declined. The best-documented example is the extinction and reintroduction of the large blue *Maculinea arion* in England (Thomas et al. 2009).

Niphanda fusca is a lycaenid butterfly distributed in Eastern Asia (Fukuda et al. 1984). This species is an ant parasite: 1st-2nd instar larvae drink honeydew of aphids on various plants, and 3rd-last instar larvae are brought into the nest of a host ant, *Camponotus japonicas*, and fed mouth-to mouth by the ant (Fukuda et al. 1984; Hojo et al. 2009). The butterfly previously had a wide geographic range throughout the Japan mainland except for Hokkaido, which consisted of many patchy and small habitats. However, it has become extinct in many areas (Mano and Fujii 2009), and is listed as Endangered (facing a high risk of extinction in the wild in the near future) in the Japan Red List (Ministry of the Environment of Japan 2012). The habitat requirement of *N. fusca* is relatively specific. *C. japonicas* builds its nests in sunny places (Imai et al. 2004), and therefore *N. fusca* also inhabits such places. Of course, *N. fusca* cannot inhabit all the places where *C. japonicas* builds its nests; for instance, it can only inhabit places where there are sufficient aphids. *N. fusca* prefers early stages of succession, such as grasslands or semi-bare areas (Fukuda et al. 1984). In the rainy climate of Japan, most semi-bare areas and grasslands become forests through succession (Kira 1971). Actually, most habitats of *N. fusca* are places where succession is prevented by human activity (Fukuda et al. 1984), such as satoyama: the traditional agricultural landscape of Japan, consisting of a mosaic of patches of forests, grasslands, ponds, and creeks (Washitani 2001). Now, many of these secondary-environment habitats have become unsuitable for *N. fusca*: such secondary environments have been destroyed or are becoming forests because they are not managed now (Mano and Fujii 2009). This declining situation is similar to that of *Maculinea* butterflies, for which habitat management is now actively performed (Thomas et al. 2009; Ugelvig et al. 2011). On the other hand, *N. fusca* also lives in natural semi-bare areas, such as cliffs in mountains or grasslands around volcanos. Since these natural-environment habitats are less affected by human life-style than habitats in secondary environments, they may be stable for much longer periods.

From the point of view of conservation strategy, it is easier to protect populations in natural environments than those in secondary environments because the existence of the latter populations depends on changeable human life-style factors that are difficult to control. Moreover, populations in natural environments may be a richer

source of genetic diversity than populations in secondary environments because the latter might have often experienced a bottleneck and a founder effect (e.g., DeChaine and Martin 2004; Neve et al. 2009). However, it is possible that *N. fusca* populations in natural environments and those in secondary environments might have been genetically differentiated, and should be treated as different evolutionarily significant units (ESU) (Crandall et al. 2000).

In this study, we investigate the genetic structure of *N. fusca* to address two questions. 1) Are populations in natural environments genetically different from those in secondary environments? and 2) Do populations in natural environments possess greater genetic diversity than those in secondary environments? For this purpose, we analyzed the distributions of mitochondrial and nuclear DNA haplotypes of this butterfly in Japan and South Korea.

Materials and Methods

Sampling protocol

We collected as samples 189 individuals of *N. fusca* from 21 sites representing the species' geographic range in Japan from 2010 to 2012, and 7 individuals from three sites in South Korea (Namyangju: 4, Yeongwol: 2, Inje: 1) in 2012 (Fig. 1). Samples were preserved in 99% ethanol or acetone at -25°C. Since *N. fusca* is an endangered species in Japan (Ministry of the Environment of Japan 2012), we need to take care not to damage its populations by our sampling. In each sampling site, we selectively collected a few older individuals per site. For additional sampling, we captured a butterfly with an insect net, and cut one middle or hind leg. Then the butterfly was marked with water-insoluble ink to avoid re-sampling, and released. We stopped taking samples when we had collected ten or more butterflies. Therefore, the sample size reflects the population size to some extent.

Extraction of genomic DNA, PCR and sequencing

Genomic DNA was extracted from individual thoraces or legs using a DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions. Fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene were amplified by polymerase chain reaction (PCR) using primer pair Ron (5'-GGATCACCTGATATAGCATTCCC-3') and Nancy (5'-CCCGGTAAAATTAAAATATAAACTTC-3') (Simon et al. 1994). Fragments of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene were PCR amplified using primer pair V1 (5'-CCTGTTTCTGCTTTAGTTCA-3') and A1

(5'-AATATDAGGTATAAATCATAT-3') (Yagi et al. 1999). Fragments of the nuclear elongation factor 1α (EF-1α) gene were PCR amplified using ef44 (5'-GCYGARCGYGARCGTGGTATYAC-3') and efrcM4 (5'-ACAGCVACKGTYTGYCTCATRTC-3') (Monteiro and Pierce 2001). Amplifications were conducted with a 3-min denaturation at 94ºC followed by 35 cycles of 0.5 min denaturation at 94ºC, 1 min annealing at 50ºC for COI and EF-1α, and 44 ºC for ND5, 1.5 min extension at 72ºC, and a final 7 min extension at 72ºC. We used rTaq or ExTaq DNA polymerase (Takara, Otsu, Japan) in a thermal cycler (Takara, Otsu, Japan). PCR products were cleaned up using ExoSAP-IT (USB Corporation, Cleveland, OH). Cycle sequencing reactions were carried out using a BigDye terminator version 3.1 (ABI) using both primers. For EF-1α, we designed an internal forward primer efNif (5'-TGCCCTGGTTCAAGGGATGG-3') because PCR products were slightly too long (ca. 1000 bp) for cycle sequencing reaction. After removing impurities, the products were sequenced using an ABI 3130*xl* sequencer (ABI). For COI and ND5, the overlapping region of each strand was used for analyses. For EF-1α, the non-overlapping region was also used because the sequence data were very clear. For EF-1α samples that contained multiple heterozygous sites, we performed TA cloning. Haplotype sequences were deposited in DNA Data Bank of Japan (Accession numbers AB844713–AB844726, LC026482–LC026491).

Data Analyses

The obtained alignment was straightforward and required no gap filling. Sequences were aligned with Clustal W2 (Larkin et al. 2007). For sampling sites with number of genes $\geq$ 10, genetic diversity within the sampling site was estimated by computing haplotype diversity (*H*) and nucleotide diversity (π) (Nei 1987) using Arlequin 3.5 (Excoffier and Lischer 2010). Haplotype diversity is the probability that two randomly sampled alleles are different, while nucleotide diversity is the average number of differences in nucleotides per site between two DNA sequences. The statistical parsimonious network was calculated using TCS version 1.21 (Clement 2000). The network was subsequently drawn by hand (Fig. 2a,b). The data from the two mitochondrial genes were concatenated for theses analyses.

We performed an analysis of molecular variance (AMOVA: Excoffier et al. 1992) implemented in Arlequin 3.5 (Excoffier and Lischer 2010) to separate *N. fusca* genetic variation into components attributable to differences among the hierarchical groups (habitat type: natural or secondary environments) (Φ_{CT}), among sampling sites within each habitat type (Φ_{SC}), and among sampling sites across the *N. fusca* distributional range (Φ_{ST}). We performed 1000 permutations under the null hypothesis

of panmixia to test significance. Since only Eastern Japan contains natural-environment habitats (Fig. 1), AMOVA including all the Japanese populations might confuse effects of habitat type and geographic signals. Therefore, we also performed AMOVA including only sampling sites in Eastern Japan (sampling sites 1~14). In addition, there may be better clustering of sampling sites than habitat type. A spatial AMOVA using SAMOVA ver. 2.0 (Dupanloup et al. 2002) was performed to identify the genetic cluster of sampling sites that maximized the F_{CT} value. We performed 100 simulated annealings for K = 2 to K = 20 partitions of sampling sites. Isolation by distance was tested by the Mantel test performed on matrices of pairwise geographic distances (ground distances) and pairwise F_{ST} values. We performed 1000 permutations to test significance using Arlequin 3.5 (Excoffier and Lischer 2010).

AMOVA, SAMOVA and the Mantel test were applied to Japanese samples because the aim of this study was to analyze the genetic structure of *N. fusca* of the Japanese archipelago. For these analyses, a combined dataset of mitochondrial DNA and EF-1α as separate loci was used

Results

We obtained the DNA sequences of 388 bp of the COI gene, 614 bp of the ND5 gene, and 654 bp of the EF-1α gene. In the data set of the two mitochondrial genes, we found 11 polymorphic sites (6 in COI and 5 in ND5) leading to 12 haplotypes (7 in COI and 6 in ND5) (Fig. 2a). The 7 alleles in the COI gene were named a~g, and the 6 alleles in the ND5 genes were named 1~6 (Fig. 2a). For the EF-1α gene, we found 7 polymorphic sites leading to 8 haplotypes (Fig. 3a)

Mitochondrial genetic diversity indices for each sampling site in the Japanese archipelago are presented in Table 1. Both haplotype diversity (H) and nucleotide diversity (π) were 0 (consisting of a single haplotype) for 11 out of 15 sampling sites with number of samples $\geq$ 10. Haplotype diversity was 0.33-0.6, and nucleotide diversity was 0.00038-0.00098, for the remaining 4 sampling sites, which contained 2 to 3 haplotypes. Of 5 natural-environment sampling sites, 3 contained a single haplotype. Of 10 secondary-environment sampling sites, 8 contained a single haplotype. A difference in haplotype diversity was not found between these two types of sampling sites (Wilcoxon rank sum test: W = 32.5, P = 0.503). Also, a difference in nucleotide diversity was not found between the two types of sampling sites (Wilcoxon rank sum test: W = 34.5, P = 0.333).

Genetic diversity indices of EF-1α for each sampling site are presented in Table 1. Both haplotype diversity (H) and nucleotide diversity (π) were 0 (samples all had a single haplotype) for 5 out of 19 sampling sites with number of samples $\geq$ 5.

Haplotype diversity was 0.1-0.63, and nucleotide diversity was 0.00015-0.0015 for the remaining 14 sampling sites, which contained 2 to 3 haplotypes. Of 5 natural-environment sampling sites, 2 contained a single haplotype. Of 14 secondary-environment sampling sites, 3 contained a single haplotype. A difference in haplotype diversity was not found between these two types of sampling sites (Wilcoxon rank sum test: W = 28.5, P = 0.575). Also, a difference in nucleotide diversity was not found between these two types of sampling sites (Wilcoxon rank sum test: W = 29.5, P = 0.64).

The AMOVA results were significant, indicating that 15.11% of the *N. fusca* genetic variation could be explained by the habitat type, and 52.93% of the genetic variation could be attributed to differences among sampling sites within each habitat type (Table 2). The result of AMOVA including only Eastern Japanese populations were also significant; however, differentiation between the habitat types was not found (Table 2). The SAMOVA results showed that our genetic data were best explained by assuming the existence of 19 groups. One group consisted of the populations in Oguni, Otari, and Higashiizu, which contained exactly the same haplotypes of mitochondrial and nuclear genes (Fig. 2b,3b). Each of the remaining sampling sites contained a different group.

The correlation between geographic distance and F_{ST} among Japanese sampling sites was significant according to the Mantel test (P = 0.027).

Discussion

Genetic diversity indices for each sampling site of *N. fusca* are lower than those of other butterflies (de Jong et al. 2011; Sielezniew et al. 2011; Downey and Nice 2013; Bossart and Antwi 2013; Sakamoto et al. 2015), and are similar to those of introduced butterfly populations (Wu et al. 2010). Some butterflies that parasitize ant nests also exhibit low genetic diversity (Ugelvig et al. 2011; Sielezniew et al. 2012; Pellissier et al. 2012). In parasitic butterflies, overlapping requirements of suitable host plants and attendant ants would lead to population fragmentation, reduced effective population size, and consequently, decrease of genetic diversity. One of our working hypotheses is that populations in natural environments would contain more genetic diversity than populations in secondary environments because the latter would have experienced more bottleneck and founder effects. However, differences in genetic diversity were not found here between natural-environment sampling sites and secondary-environment sampling sites. At this stage, therefore, the low genetic diversity in *N. fusca* should be attributed to their obligate parasite life history, rather than habitat loss caused by change of human life-style.

Habitat type explained 15.11% of *N. fusca* genetic variation. This result seems to indicate that populations in natural environments and those in secondary environments are somewhat differentiated. However, all five natural-environment habitats studied here were located in Eastern Japan (Fig. 1), and the AMOVA results may reflect a local population divergence. In fact, when we performed AMOVA including only sampling sites in Eastern Japan, habitat type could not explain *N. fusca* genetic variation (Table 2).

52.93% of the genetic variation was found to arise within the same habitat type, indicating that factors other than habitat type have a major effect on *N. fusca* geographic variation. In the present case, the result of the Mantel test was significant, indicating that geographic distance plays a role. Potential factors that could have caused the geographic variation include an effect of symbiosis. Host ants may play a role in the divergence of butterflies (Als et al. 2004; Eastwood et al. 2006). In addition, it is known that symbiotic bacteria such as *Wolbachia* can also affect the genetic structure of their host insects through a selective sweep (e.g. Narita et al. 2006; Graham and Wilson 2012; Sielezniew et al. 2012). Further research is needed to clarify these effects.

Our data did not provide a strong reason to consider natural-environment populations and secondary-environment populations as different ESUs. The SAMOVA results indicated that *N. fusca* cannot be clustered genetically in a few large groups, although each sampling site exhibits genetic differences. Perhaps this is because the genetic differentiation of each sampling site is limited. However, it should be noted that this study used only genetic markers. Phenotypic adaptations may occur in both types of environments.

N. fusca is already endangered in Japan (Ministry of Environment of Japan 2012), and its conservation must be initiated as soon as possible. At present, we think it is practical to conserve the butterflies in natural environments at first, because in that case, we need not manage habitats to protect *N. fusca* for the time being. We have only to prevent habitat destruction. In contrast, in order to conserve the butterflies in secondary environments, we would have to continue managing the habitats. This is far more difficult than preventing habitat destruction because it requires that we place sufficient new value on the traditional environmental management to continue it (Washitani 2001). It is fortunate for *N. fusca* that there exist natural-environment habitats, although their number is limited (Fig. 1). In addition, we were able to collect ten or more samples in all of the five natural-environment sampling sites, while we could not collect ten samples in six secondary-environment sampling sites (Table 1), suggesting that the population sizes of natural-environment habitats are relatively larger. Some butterfly species living only in secondary grasslands in Japan, such as

Shijimiaeoides divines, *Melitaea protomedia*, and *Fabriciana nerippe*, are experiencing much more severe declines because there are no remaining habitats for them when secondary grasslands are abandoned or destroyed (Nakamura 2011).

Of course, we do not think that it is impossible to maintain habitats in secondary environments. Conservation of secondary-environment habitats could be successful in habitats that contain many endangered species and that are considered valuable by many people.

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Table 1 The mitochondrial and nuclear gene diversity index of each sampling site. Number of individuals, haplotype diversity (*H*) ± SD and nucleotide diversity (π) ± SD of each site are shown. Location numbers are identical to those in Fig.1

			mitochondrial DNA		nuclear DNA	
Location	**Habitat**	**Number of individuals**	***H*** (± SD)	**π** (± SD)	***H*** (± SD)	**π** (± SD)
1. Oguni	secondary	7			0	0
2. Uonuma	natural	10	0.4667 ± 0.1318	0.000466 ± 0.0005	0.6158 ± 0.077	0.001086 ± 0.000951
3. Shirosato	secondary	13	0	0	0.1732±0.1009	0.000265±0.000406
4. Otari	secondary	10	0	0	0	0
5. Fujikawaguchiko	natural	11	0	0	0.4978±0.1022	0.000872±0.000793
6. Higashiizu	secondary	3				
7. Nakatsugawa	secondary	5			0.5333±0.0947	0.000815±0.000833
8. Ohno	natural	10	0	0	0	0
9. Higashiohmi	natural	11	0.3273 ± 0.1533	0.00098 ± 0.000808	0	0
10. Miyagawa	natural	11	0	0	0.1732±0.1009	0.000265±0.000406
11. Nara	secondary	10	0	0	0.3368±0.1098	0.000515±0.000596
12. Totsugawa	secondary	10	0	0	0	0
13. Sanda	secondary	10	0	0	0.1000±0.0880	0.000153±0.000303
14. Toyooka	secondary	13	0.3846 ± 0.1321	0.000384 ± 0.000433	0.5942±0.0537	0.001019±0.000904
15. Maniwa	secondary	11	0	0	0.6277±0.0602	0.001119±0.000965
16. Nishinoshima	secondary	12	0	0	0.5543±0.0872	0.001097±0.000949

17. Akiohta	secondary	5			0.6000±0.1305	0.001529±0.001267
18. Umi	secondary	10	0.6 ± 0.1305	0.000665 ± 0.000628	0.5053±0.0560	0.000773±0.000763
19. Higashisonogi	secondary	5			0.5333±0.0947	0.000815±0.000833
20. Takamori	secondary	10	0	0	0.1000±0.0880	0.000153±0.000303
21. Tarumizu	secondary	2				

Table 2 Analyses of molecular variance (AMOVA) for grouping by habitat type

	d.f.	Sum of Squares	Variance component	Percentage of variation	Fixation Index	P
Japan						
among G	1	19.95	0.0867 Va	15.11	Φ_{CT}: 0.15113	0.03128
among P	19	105.419	0.31058 Vb	52.93	Φ_{SC}: 0.62355	< 0.00001
within P	347	65.063	0.18750 Vc	31.96	Φ_{ST}: 0.68044	< 0.00001
Easten Japan						
among G	1	17.204	0.0751Va	12.56	Φ_{CT}: 0.12556	0.09286
among P	12	84.552	0.37995Vb	63.52	Φ_{SC}: 0.76077	< 0.00001
within P	244	34.914	0.14309Vc	23.92	Φ_{ST}: 0.72642	< 0.00001

Fig. 1 The locations of the sampling sites. Open circles indicate natural environments, and filled circles indicate secondary environments. Sampling sites are indicated by the city or town name. 1. Oguni, 2. Uonuma, 3. Shirosato, 4. Otari, 5. Fujikawaguchiko, 6. Higashiizu, 7. Nakatsugawa, 8. Ohno, 9. Higashiohmi, 10. Miyagawa, 11. Nara, 12. Totsugawa, 13. Sanda, 14. Toyooka, 15. Maniwa, 16. Nishinoshima, 17. Akiohta, 18. Umi, 19. Higashisonogi, 20. Takamori, 21. Tarumizu, 22. Namyangju, 23. Yeongwol, 24. Inje

Fig. 2 (**a**) A haplotype network of mitochondrial genes. Each node in the haplotype network represents a single nucleotide change and each branch represents a single mutational step. The alphabetical part of the haplotype names indicates the COI allele, and the numerical part of the haplotype names indicates the ND5 allele. Circle areas in the haplotype network are proportional to observed numbers of haplotype copies present in all the samples.

(**b**) Distribution of mitochondrial haplotypes Haplotypes shared by at least two sampling sites are indicated by the same pattern, and unique haplotypes present only in one specific site are indicated by the haplotype names.

Patterns and names for each haplotype are the same in (**a**) and (**b**).

Fig. 3 (**a**) A haplotype network of the nuclear gene.

(**b**) Distribution of nuclear haplotypes.

Fig. 1

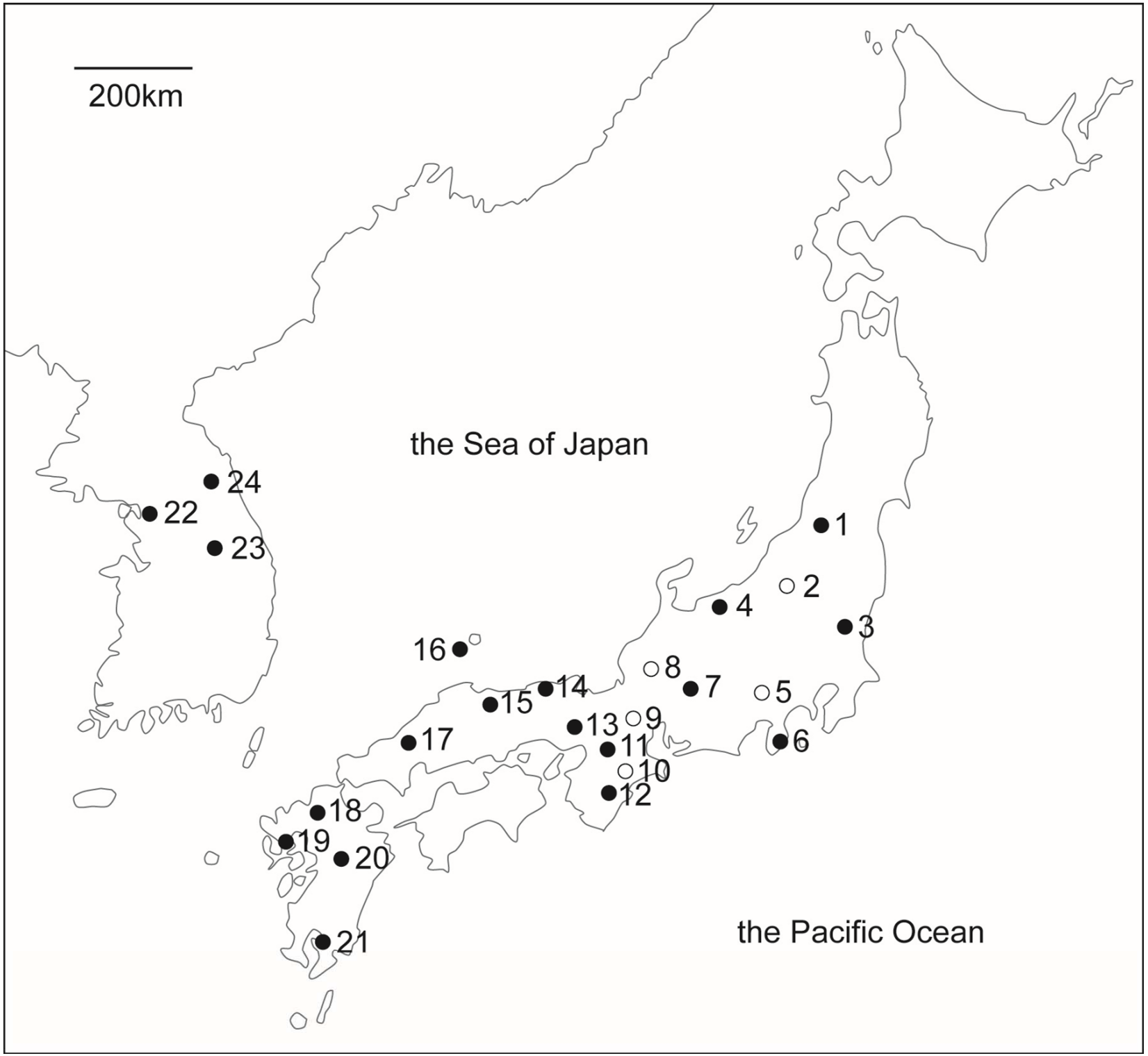
200km
the Sea of Japan
the Pacific Ocean
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Fig. 2(a)

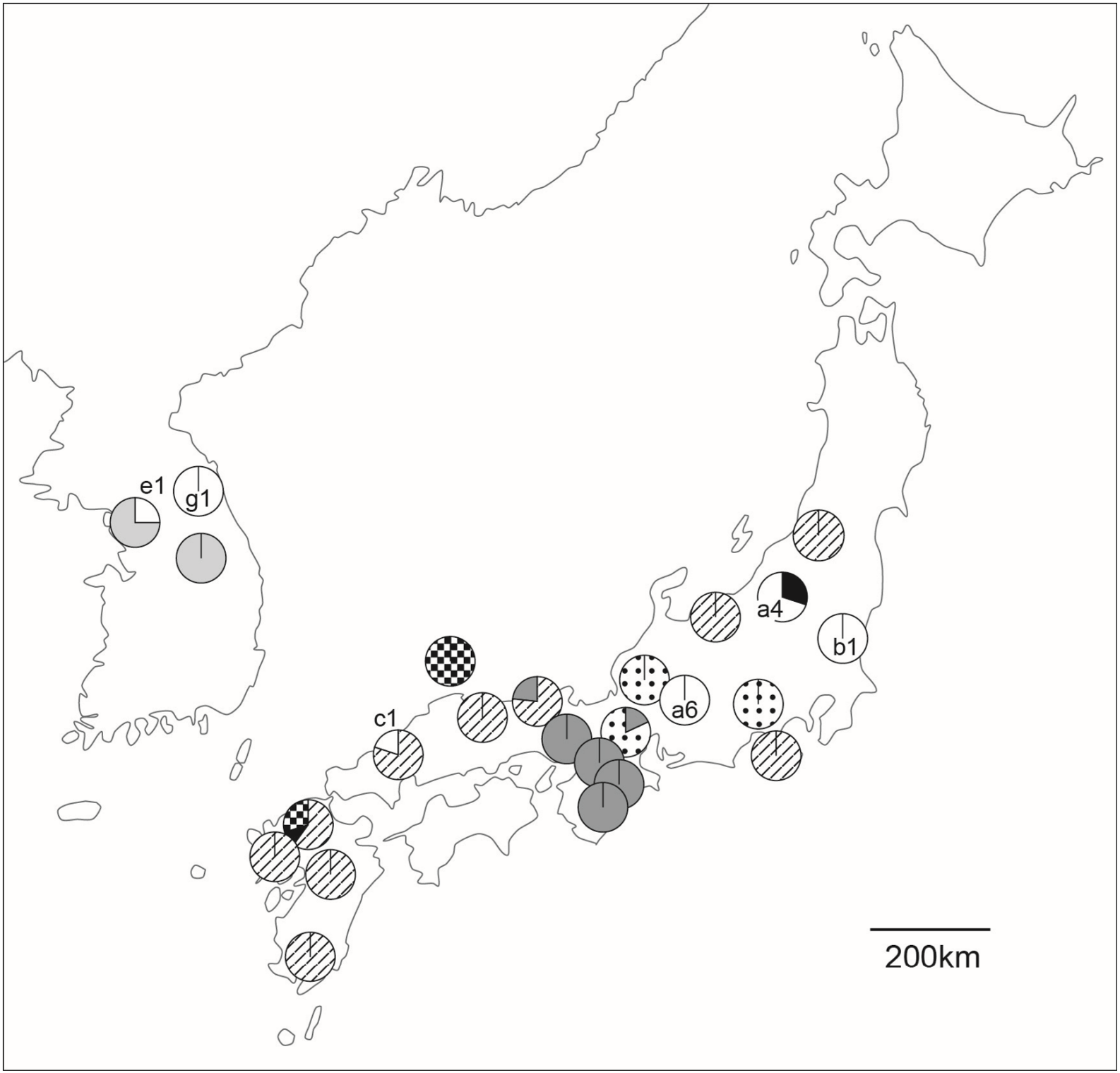

Fig. 2(b)

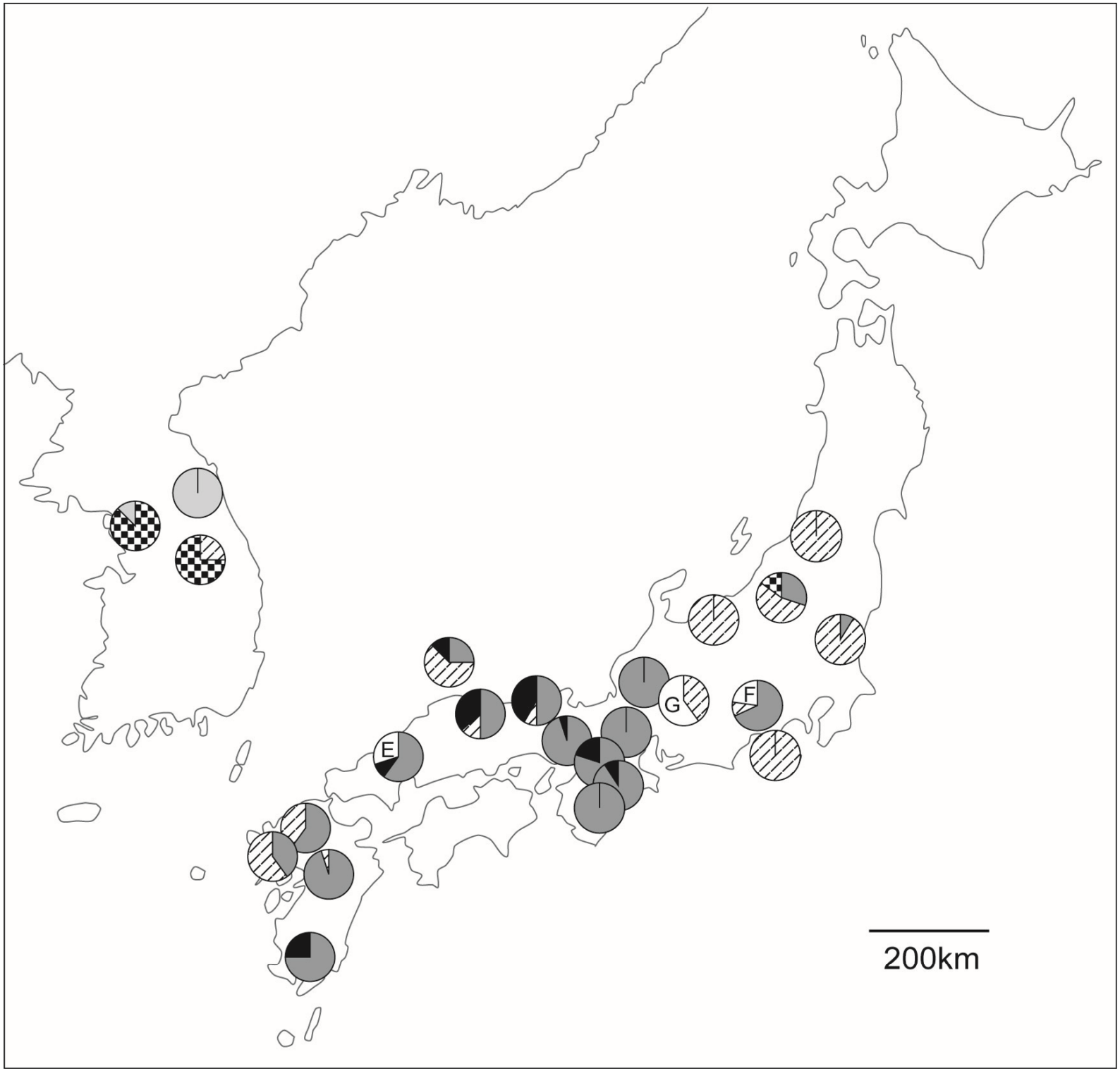

Fig. 3(a)

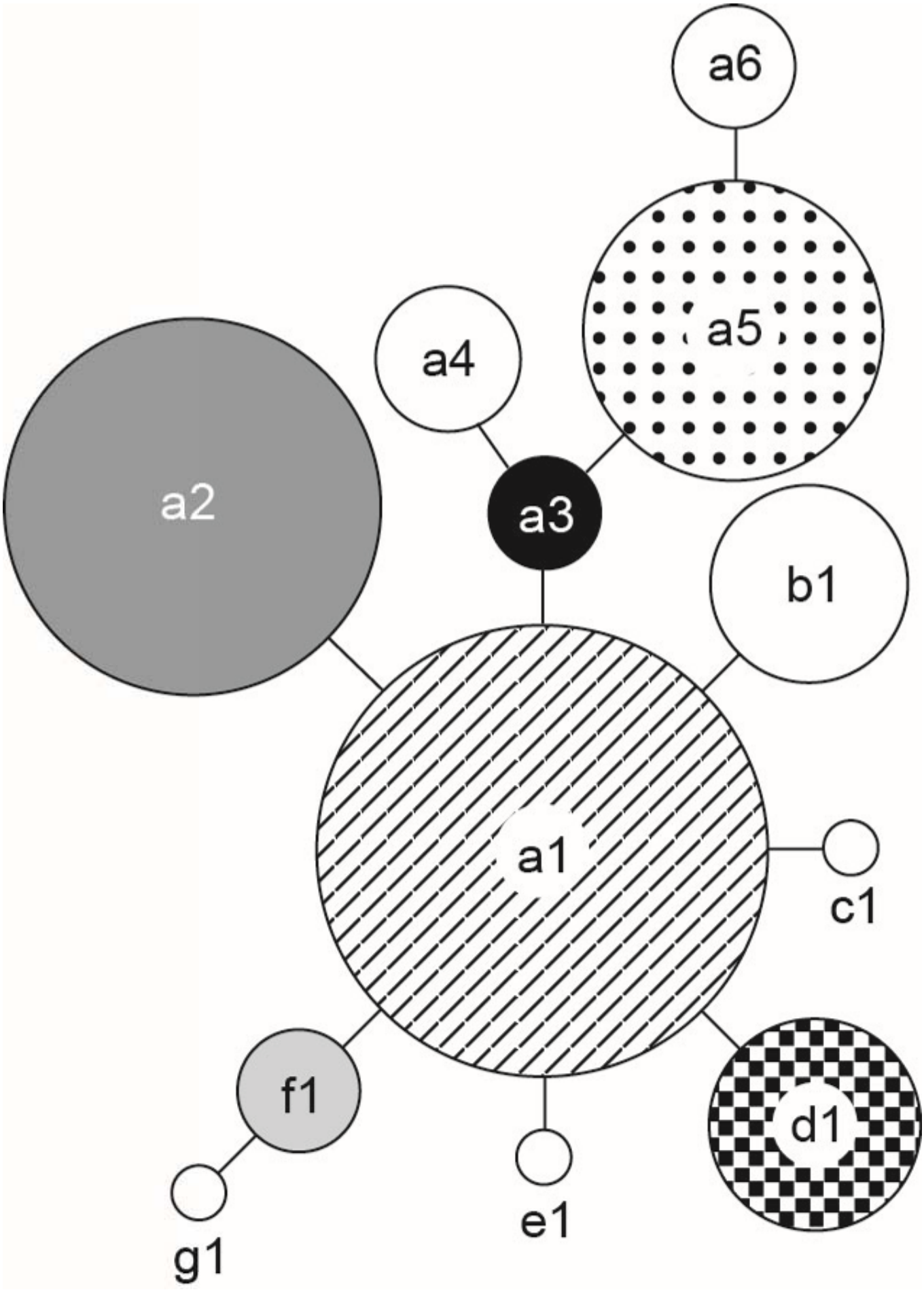

Fig. 3(b)